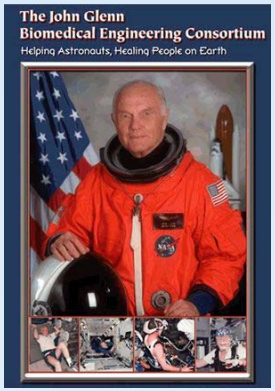

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Status Report

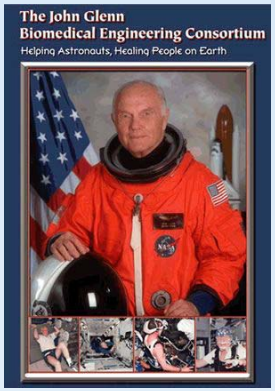
In Vivo Bioluminescent Molecular Imaging with Application to the Study of Secretory Clusterin, a Potential Biodosimeter during Space Exploration

PI: David L. Wilson

Glenn Research Center

at Lewis Field

NASA



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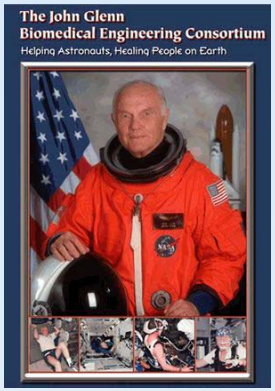
Accomplishments

- We determined the optimal range of cell number for experiment measurements of pelleted cells in a 96-well plate.
- Dose response experiments were performed of the MCF7-1403 breast cancer cells using optimized cell numbers.
- Dose response experiments were also performed with the cells attached to the well plate.



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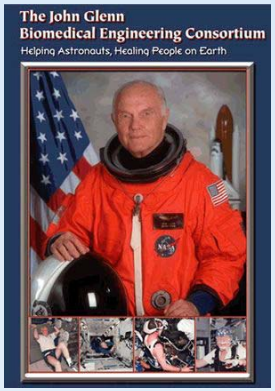
Progress versus Scheduled Activities

- We are on schedule.



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Determination of optimal cell number for measurements of pelleted cells in 96-wellplates

Our earlier data indicates that as we increase the amount of luciferase, the amount of light detected increases linearly assuming luciferin is not a limiting reagent. But when experiments were conducted with higher cell concentration, the intensity of light detected was reduced (FIG.1). This occurred in both the untreated (UT) and the irradiated (IR) sample.

This prompted us to investigate into determining an optimal cell number for further experiments in 96 well plates. We believe this phenomenon occurs because higher concentration of cells in the well plate, prevents light from underlying cells from being detected by the CCD camera.

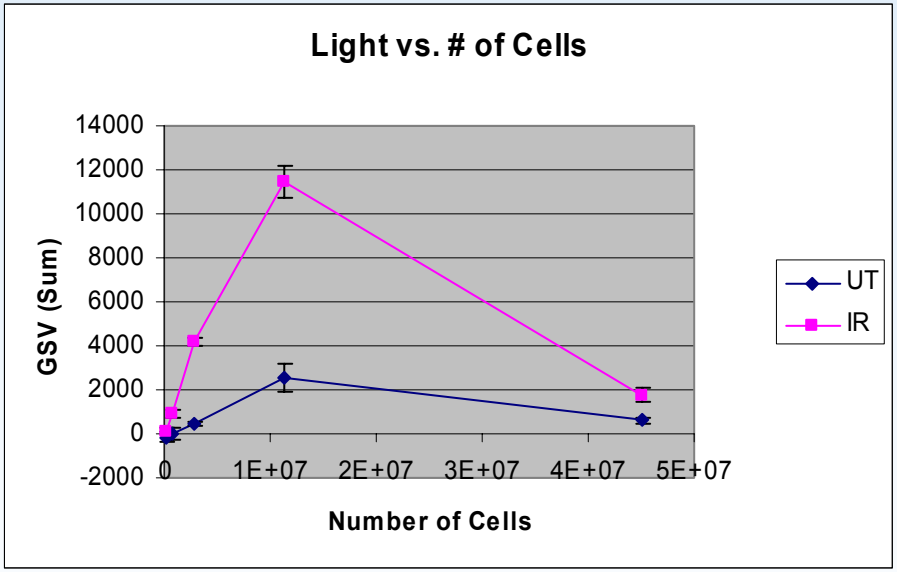

FIG:1

Results: We are confining our experiments to a maximum of 10^6 cells per well in a 96-well plate.

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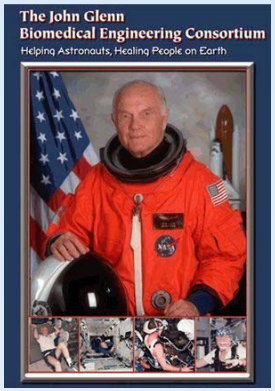

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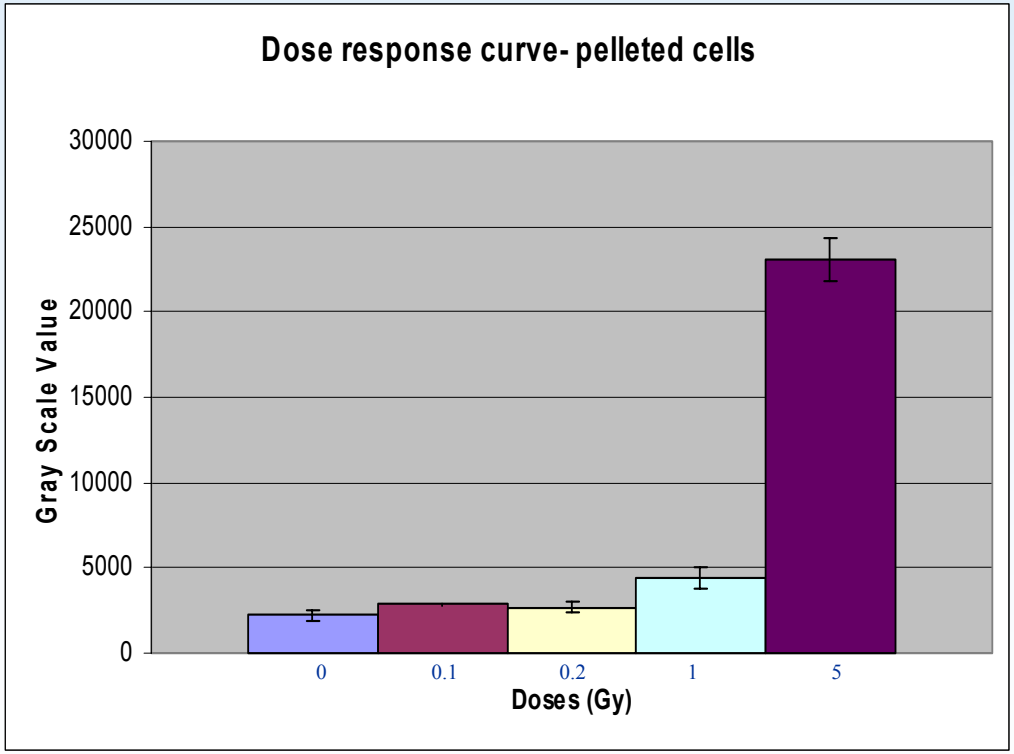
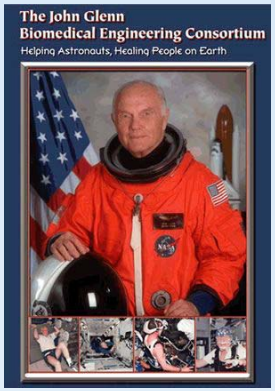
Dose response curves with pelleted cells

Rationale: Experiments with pelleted cells are advantageous compared to attached cells because we can enforce a strict control on the number of cells taken for analysis and hence a more accurate assessment of untreated (UT) vs irradiated (IR) can be done. This is because IR treatment leads to cell cycle arrest and growth inhibition causing different count of cell numbers during the time of imaging.

Result: We observed a steady increase in clusterin expression with increase in radiation dose. We also noticed a difference in detected light at low radiation doses.





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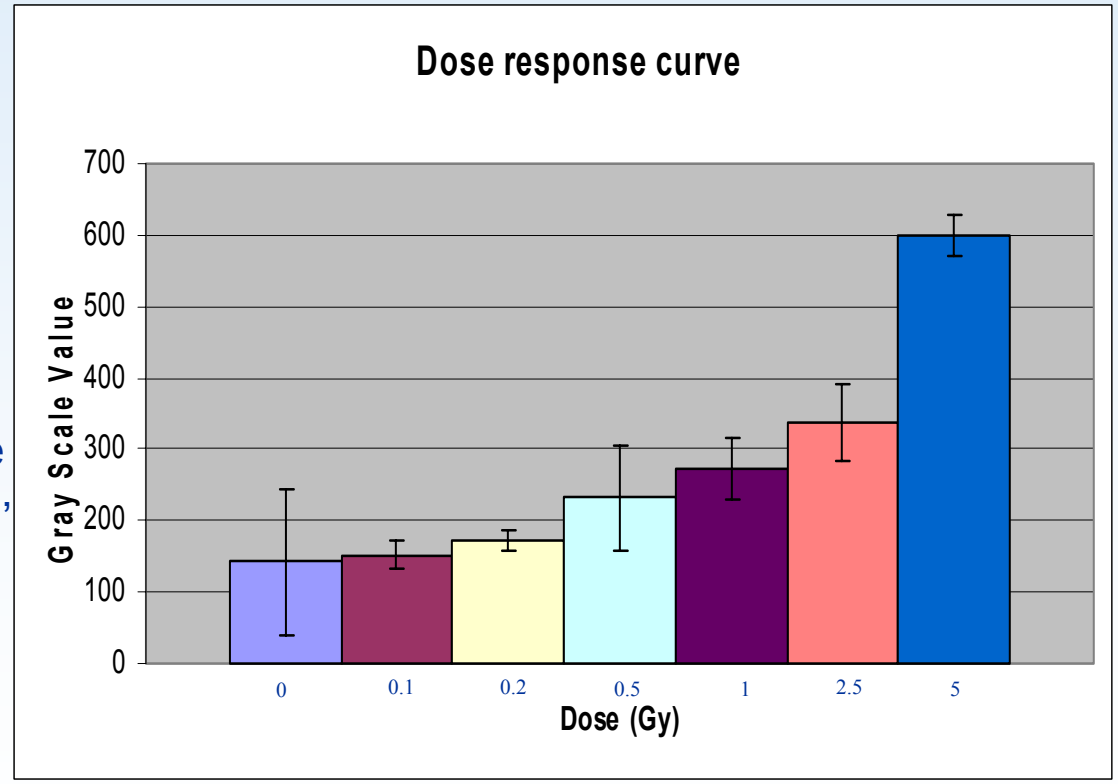
Dose responses with attached cells

Rationale: In spite of the fact that pelleted experiments are more accurate in recording accurate cell numbers, procedures of cell harvesting, counting, pelleting and resuspension in Luciferin Assay Reagent (LAR) may affect proper luciferase detection, so it would be useful to have an idea of what is happening in attached intact cells. We have made efforts to control the cell numbers so that during imaging, the number of cells per dose are almost equal.

Result: Attached cells also show an increase in clusterin expression with increase in radiation dose.



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In vivo bioluminescent imaging for quantifying gene expression

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Department of Biomedical Engineering, Case Western Reserve University

ABSTRACT

In vivo bioluminescent imaging (BLI) is a technique which utilizes biological molecules as a source for image contrast. We used BLI to evaluate the *in vivo* gene expression from intradermal injections of uncompacted and compacted plasmids that contained the reporter gene for firefly luciferase. Luciferin, a probe molecule, reacts in the presence of luciferase and produces photons of light which can be measured by the BLI system.

First, the response of the BLI was compared to the luminometer, a device typically used for luciferase activity measurements. Increasing concentrations of recombinant luciferase were mixed with a luciferin solution and the resulting light was measured. We found a linear relationship between the responses of the two systems. Second, we examined the *in vivo* dose-response of intradermally injected uncompacted and compacted DNA in rats for the two systems. BLI measurements were made by anesthetizing the rat, injecting luciferin, and imaging for six minute intervals. Luminometer measurements were made by euthanizing the rat, harvesting and lysing the skin tissue, and measuring the luciferase activity. We found that there was correlation between the response of the two systems.

Bioluminescent imaging provides a more efficient method to quantify *in vivo* gene expression. Results can be obtained within minutes without the lengthy process of preparing tissue. Also, the same animal can be studied over a course of time since euthanization is not necessary with BLI.

INTRODUCTION

Reporter genes encoding light-emitting proteins genes such as the luciferase enzyme are commonly used to study gene expression. Instruments such as luminometers and photomultiplier tubes are typically used to evaluate the expression from these reporter genes

In this study, we utilize the luciferase/luciferin reaction that occurs in fireflies. Expressed luciferase protein combined with our probe molecule, luciferin, along with ATP, magnesium, and O_2 produces light. The wavelength of this light produced is on the order of 560 – 620 nm.

The conventional method to study luciferase activity requires a lengthy process. This process includes euthanization of the test subject, taking a biopsy of the area of interest, grinding and lysing the tissue, and then measuring the light output on a luminometer.

Bioluminescent imaging (BLI) provides an alternative method to the study of biological activity in an actual *in vivo* setting due to the semi-transparent properties of mammalian tissue. Since mammalian tissue is only semi-transparent only a small amount of light generated from the light emitting probes actually penetrates the tissue. Utilizing a highly sensitive, liquid nitrogen cooled, charged-coupled device (CCD) allows us to measure the low light levels penetrating the tissue without having to go through to process of excising tissue.

OBJECTIVE

Demonstrate that BLI is an efficient tool for studying *in vivo* gene expression and gives results comparable to the conventional method for quantifying gene expression.

BIOLUMINESCENT IMAGING SYSTEM

The bioluminescent imaging system consists of a charged-coupled device (CCD) camera, a light-tight enclosure, a controller, and a computer (Figure 1).

Selection of a CCD camera is crucial to the *in vivo* applications of the imaging system. The camera must have the ability to detect low light levels while minimizing read noise. Another important factor in the selection of the CCD camera is the quantum efficiency, the probability that a photon of light striking the detector is converted into a signal. Quantum efficiency is dependent on the wavelength. For this imaging system, we selected the Roper Scientific Versarray 1024 liquid nitrogen cooled CCD camera. This camera offers a very low read noise of approximately 4 electrons/pixel/sec while maintaining a high quantum efficiency of ~ 90% for wavelengths of 560–620 nm. Coupled to this camera is a Nikon 50 mm lens for focusing purposes.

The camera is mounted and sealed on top of the light-tight enclosure. In order to make the door of the enclosure light-tight, a double baffle seal is used to minimize light penetration into the box. To supplement the seal, a heavy dark sheet is used to cover the system during imaging sessions. The inside of the box contains lights utilized for taking photographic images, as well as an adjustable stage to vary the field of view.

Imaging parameters such as integration time and binning are changed through the computer and controller.

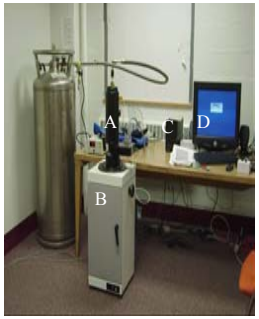

Figure 1 – Bioluminescent Imaging System. The biolumin-escent imager consists of four different components: Cooled CCD Camera (A), Light-tight enclosure (B), Controller (C), and Computer (D). The CCD camera is cooled by liquid nitrogen.

CALIBRATION

Calibration is necessary to compare results from the bioluminescent imaging system to those obtained from a conventional luminometer. To accomplish this, we used two calibration methods. The first method utilized an *in vitro* luciferase assay, and the second method utilized *in vivo* injections of plasmid DNA encoding luciferase.

In the first calibration method, solutions of recombinant luciferase protein (5-500 pg/ml) were used to evaluate the responses of the imaging system and the luminometer. Luciferase assay reagent (LAR), which contained all the components necessary for the luciferase/luciferin reaction, was added to the luciferase solution. For the imaging system, the luciferase and LAR were mixed in a black 96-well plate, and the plate was immediately imaged to quantify the luciferase activity. The calibration was also performed in small tubes and quantified using a luminometer. From these calibrations a standard curve was generated for both the imaging system and the luminometer (Figure 2).

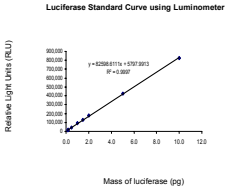

Figure 2 – Luciferase Standard Curves: The units of measure for the luminometer and the imaging system are relative light units (RLU) and gray scale values respectively. Integrated Gray Scale Values are obtained by taking a region of interest (ROI) around each luminescent site, subtracting the read noise, and adding up all of the pixel values in the ROI.

A rodent model of skin gene therapy was used for the *in vivo* calibration. Sprague Dawley rats were injected intradermally with 1-50 µg of a plasmid encoding luciferase. The plasmid was either compacted with a polylysine-polyethylene glycol conjugate or uncompacted. After 24 hours, luciferin (126 mg/kg) was injected into the animal subjects and imaging was performed. First, a photographic image of the animal subject was taken using the lights inside the light-tight enclosure. Then the lights were turned off, and a six minute exposure image of the luminescence was taken. After imaging, the animals were euthanized, and skin biopsies from the injection sites were removed and lysed. The lysate was mixed with LAR, and the luciferase activity was measured in the luminometer. Using the luminometer standard curve developed from the *in vitro* calibration, the mass of luciferase produced *in vivo* was calculated. These values were plotted against the gray scale value obtained from the imaging system (Figure 3).

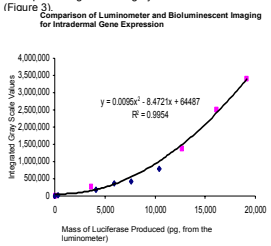

Figure 3 – In vivo comparison: Luciferase mass was calculated using the luminometer standard curve generated by the *in vitro* calibration. These luciferase masses were plotted versus imaging system values and a power relationship was found between the two systems.

The power relationship between the two systems shows a high correlation with the imaging system showing a higher sensitivity than the luminometer.

IN VIVO BIOLUMINESCENT IMAGING OF GENE EXPRESSION

After calibrating the imaging system and verifying that it could detect luciferase activity *in vivo*, the time course of gene expression in rodent skin was evaluated over 14 days. Each test subject was injected with uncompacted and compacted DNA, as well as saline solution for a negative control. Injections were intradermal and were approximately 1-2 mm beneath the surface of the skin.

The animals were imaged on days 1, 2, 4, 7, and 14. Bioluminescent imaging was performed as described for the calibration experiments, with a photographic image being acquired first and then bioluminescent images. Images were recorded over a time course of approximately 60 minutes with each image requiring 6 minutes.

Using a pseudo color overlay program written in Matlab, the integrated bioluminescent images were converted into a color scale and superimposed on top of the black and white photographic images (Figure 4).

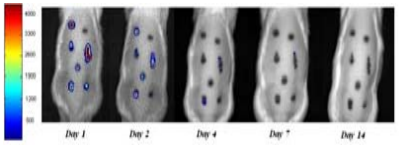

Figure 4 – Sustained Gene Expression: Injections of compacted and uncompacted DNA were placed 1-2 mm under the skin of the rats. Bioluminescent images were windowed and leveled so that the highest bioluminescent signal would be represented by red and the lowest signal represented by dark blue.

On day 1, the pseudo colored images showed that all injection sites, except for the negative control, were producing luciferase. Day 2 showed a reduction in luciferase activity at each injection site. Some injection sites did not have significant luminescence. On days 4 and 7, only a few sites had luminescence that was detected by the imaging system. By day 14, no injection sites showed luminescence. In addition, we also noticed that each bioluminescent injection site had a higher luminescence near the center of the injection site and decreased moving outward.

DISCUSSION

It has been shown that intradermal injections of plasmid DNA usually produce gene expression that peaked within 24 hours and was undetectable within 7 days. Our results from the bioluminescent imaging system also demonstrate that gene expression in the skin lasts approximately a week. On day 7, only one injection site produced detectable luminescence, and on day 14 no injection sites produced luminescence. We also noticed that the bioluminescent images presented a signal for each injection site that was highest in the center and decreasing radially outward. This is explained by the semi-transparent properties of mammalian tissue which cause absorption and scattering of the light as it penetrates through the tissue. A penetration depth of approximately 1-2 mm can result in a scattering of up to 2-3 mm in any direction from the source. Although results from the bioluminescent imaging system also demonstrate that gene expression in skin lasts approximately one week, a small number of rodent models were used. We are continuing to study the effects of intradermal injections of plasmid DNA in additional rodent models and are also studying the effects of a non-viral gene delivery vehicle.

CONCLUSIONS

In vivo bioluminescent imaging is a promising technology in the study of gene expression, as it provides a quick and efficient method for obtaining results. The semi-transparent properties of mammalian tissue and the development of highly sensitive CCD cameras make bioluminescent imaging possible. Time is saved through bioluminescent imaging with the ability to obtain results within minutes in comparison to several hours required for preparing tissue for the luminometer. In addition, serial animal sacrifice is not necessary and the same animal can be studied over a course of time, reducing the number of animals required to complete the study (Figure 5). This also reduces patient variability. The expression of uncompacted and compacted plasmid DNA over a 14 day time course has been observed through bioluminescent imaging, demonstrating the potential of this type of imaging as an *in vivo* technology in the study of gene expression.

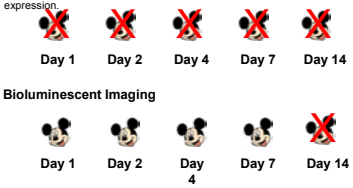

Figure 5 - Fewer Animal Sacrifices: Conventional methods to measure luciferase require an animal subject to be euthanized for every time point data is collected. This requires many animal subjects for long term experiments. Bioluminescent imaging offers an alternative solution where a single animal subject can be studied over a course of time.

ACKNOWLEDGEMENTS

The creation of the bioluminescent imaging system was funded with a grant from the John Glenn Biomedical Engineering Consortium, a NASA group, to Dr. Wilson. In addition, this research was supported by the NSF grant CAREER-BES9874631 to Dr. Bellamkonda.

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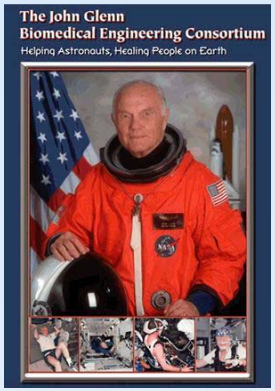

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Near-term Future Work:

- Time-Course experiments of 1403 cells to determine an optimal time to conduct experiments after irradiation.

Schedule Updates/revisions:

- None at this time